Cell

EXTENDED EXPERIMENTAL PROCEDURES

The NUPACK server (Zadeh et al., 2011) was used to predict thermally stable DNA sequences lacking secondary structures to make dsDNA bridges. Bridge and linker sequences are listed in Table S6.

IgG Heterodimers

Bispecific IgGs were constructed using "knobs-into-holes" mutations (Thr366Trp on one heavy chain, and Thr366Ser, Leu368Ala, and Tyr407Val on the other heavy chain [Atwell et al., 1997]) to promote Fc heterodimerization, and crossover of the heavy and light chain domains of one half of the bispecific IgG to prevent light chain mispairing (Schaefer et al., 2011). Heterodimerizing leucine zipper sequences (O'Shea et al., 1993) followed by either a 6x-His or Strep II tag sequence (Schmidt and Skerra, 2007) were added to the C-termini of the heavy chains. The V_H domain on one heavy chain of each heterodimer was replaced by the V_L domain, and the corresponding light chain was constructed with the V_H domain joined to the C_L domain as described (Schaefer et al., 2011). Heterodimeric IgGs were expressed by transient transfection and isolated from supernatants by Protein A chromatography followed by Strep II and Ni-NTA chromatography. Heterodimers were further purified by size exclusion chromatography using a Superdex 200 10/300 or 16/600 column (Amersham Biosciences) equilibrated in PBS pH 7.4.

In Vitro Neutralization Assays

Neutralization of pseudoviruses derived from primary HIV-1 isolates was monitored by the reduction of HIV-1 Tat-induced luciferase reporter gene expression in the presence of a single round of pseudovirus infection in TZM-bl cells as described (Montefiori, 2005). In some cases, DEAE-dextran, an additive used to enhance viral infection of target cells (Montefiori, 2005), led to false positive neutralization signals for dsDNA alone and for dsDNA-containing reagents, presumably because of interactions between dextran and DNA (Maes et al., 1967). Dextran was eliminated from assays in which the dsDNA linker alone reduced infectivity, in which case the pseudovirus concentration was increased by 2.5-40–fold, allowing for comparable infectivity as in the presence of dextran.

Pseudoviruses were generated by co-transfecting HEK293T cells with vectors encoding Env and a replication-deficient HIV-1 backbone as described (Montefiori, 2005) or obtained from the Fraunhofer Institut IBMT (6535.3, CAAN5342, CAP45, CAP210.200.E8, DU172, DU422, QH-0692, THRO4156.18, TRO.11, ZM53, ZM214, ZM233, ZM249). Neutralization assays were preformed in-house (Figures 2, 3, and 4) and by the Collaboration for AIDS Vaccine Discovery (CAVD) core neutralization facility for testing against a panel of isolates (Figure 2C and Tables S1, S2, S3, S4, and S5). Some of the in-house data were derived from neutralization assays that were prepared by a Freedom EVO (Tecan) liquid handler. Reagents (prepared as 3-, 4-, or 5-fold dilution series; each concentration in duplicate or triplicate) were incubated with 250 (when DEAE-dextran was added) or > 1000 viral infectious units at 37°C for one hour prior to incubation with reporter cells (10,000/well) for 48 hr. Luciferase levels were measured from a cell lysate using an Infinite 200 Pro microplate reader (Tecan) after addition of BrightGlo (Promega). Data were fit by Prism (GraphPad) using nonlinear regression to derive IC_{50} values. IC_{50}s derived from independent replicates of manual and robotic assays generally agreed within 2-4 fold. Average IC_{50} values reported in the figures and tables are geometric means calculated using the formula $(\prod a_i)^{(1/n)}$; $i = 1, 2, \ldots, n$. Geometric means are suitable statistics for data sets covering multiple orders of magnitude (Sheskin, 2004), as is the case for neutralization data across multiple viral strains. Fold improvements were calculated as the ratio of the geometric mean IC_{50} values for the reagents being compared.

SUPPLEMENTAL REFERENCES

Atwell, S., Ridgway, J.B., Wells, J.A., and Carter, P. (1997). Stable heterodimers from remodeling the domain interface of a homodimer using a phage display library. J. Mol. Biol. *270*, 26–35.

Maes, R., Sedwick, W., and Vaheri, A. (1967). Interaction between DEAE-dextran and nucleic acids. Biochim. Biophys.ACTA *134*, 269–276.

O'Shea, E.K., Lumb, K.J., and Kim, P.S. (1993). Peptide 'Velcro': design of a heterodimeric coiled coil. Curr. Biol. *3*, 658–667.

Schmidt, T.G., and Skerra, A. (2007). The Strep-tag system for one-step purification and high-affinity detection or capturing of proteins. Nat. Protoc. *2*, 1528–1535.

Sheskin, D. (2004). Handbook of Parametric and Nonparametric Statistical Procedures, Third Edition (Boca Raton: Chapman & Hall/CRC).

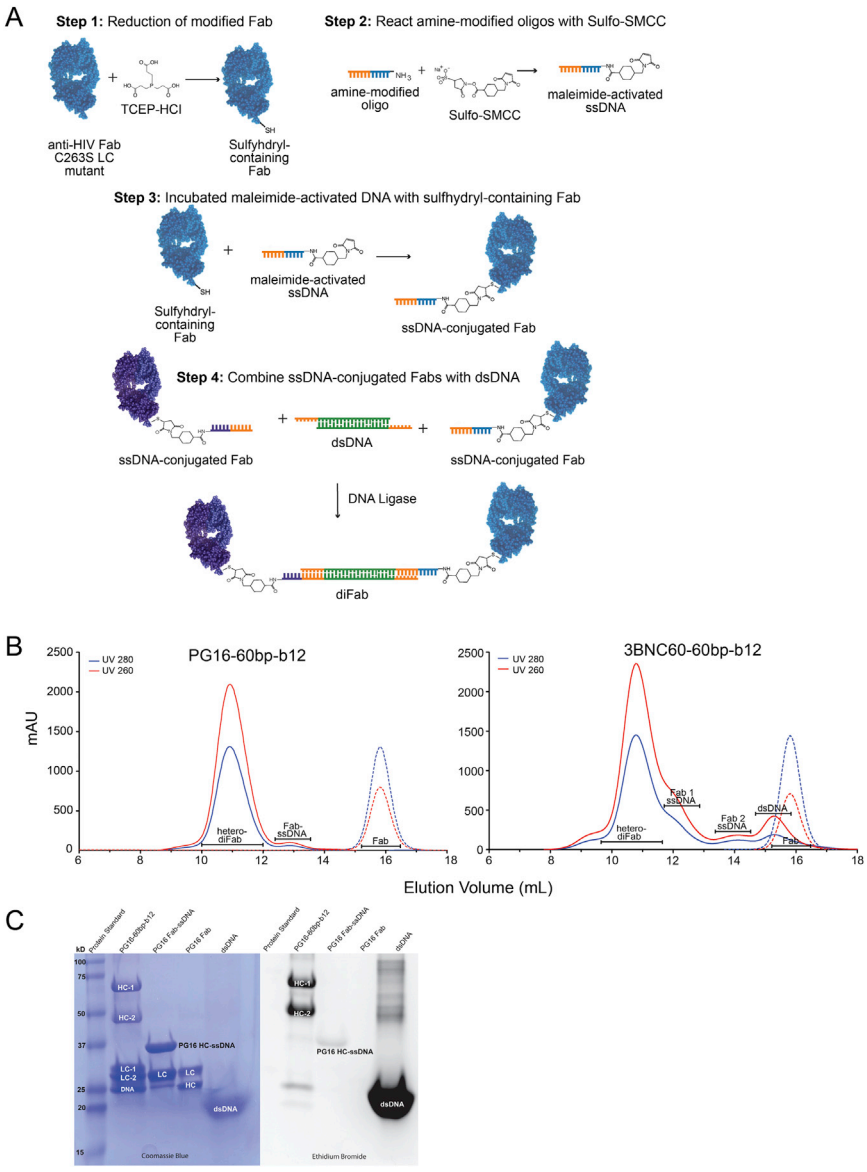

Figure S1. Production of Fabs Connected with dsDNA Linkers, Related to Figure 1

(A) Schematic of method to make homo- and hetero-diFabs. Step 1: Mild reduction of Fab containing a free thiol group at C terminus of the heavy chain. Step 2: An amine-modified ssDNA oligonucleotide is reacted with Sulfo-SMCC (amine-to-sulfhydryl crosslinker) to form a maleimide-activated ssDNA. Step 3: The reduced Fab and maleimide-activated ssDNA are incubated to form a Fab conjugated to ssDNA. Step 4: Two ssDNA-conjugated Fabs (identical Fabs for making homo-diFabs; different Fabs for making hetero-diFabs) are joined with a dsDNA containing overhangs complementary to the ssDNA, and then ligated to form a homo- or hetero-diFab.

(B) Size exclusion chromatography profiles for hetero-diFabs. Examples from which PG16-60bp-b12 (left) and 3BNC60-60bp-b12 (right) hetero-diFabs were isolated are shown (solid red line: A_{260}; solid blue line: A_{280}). The migration of a Fab that was not linked to DNA is shown for comparison (dashed red line: A_{260}; dashed blue line: A_{280}).

(C) SDS-PAGE analysis for PG16-60bp-b12 purification. Size exclusion chromatography fractions were assayed by 10% SDS-PAGE (stained with Coomassie Blue for protein or with ethidium bromide for DNA).

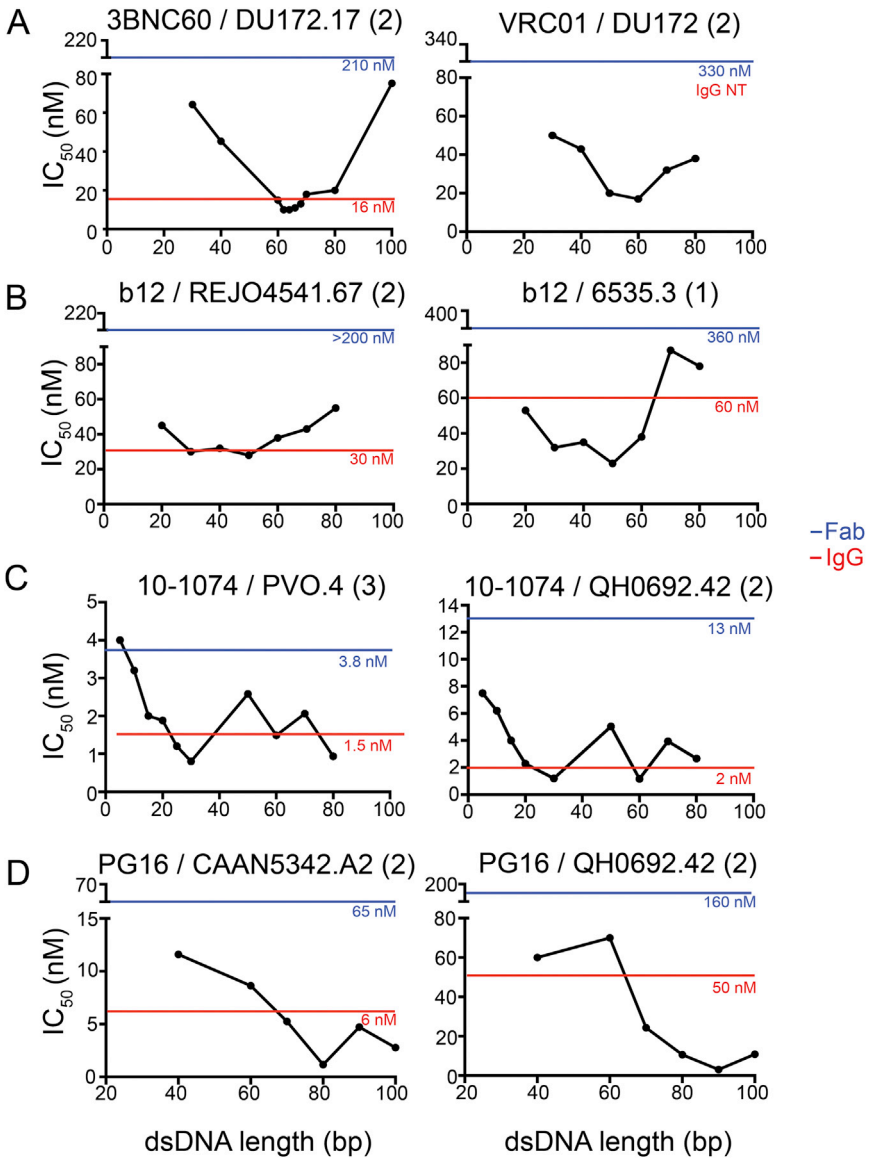

Figure S2. Effects of dsDNA Bridge Length on Neutralization Potencies of Homo-diFabs, Related to Figure 2

IC_{50}s for neutralization by homo-diFabs of the indicated HIV-1 strains plotted against the length of the dsDNA linker. In each plot, the Fab in the homo-diFab is listed before the viral strain (Tier classification (Seaman et al., 2010) included in strain name) against which the reagents were evaluated. IC_{50}s for the analogous IgG and Fab are indicated as red (IgG) and blue (Fab) lines. NT (not tested) indicates an IC_{50} that was not derived.

(A) CD4bs homo-diFabs.

(B) b12 homo-diFab.

(C) 10-1074 homo-diFab.

(D) PG16 homo-diFab.

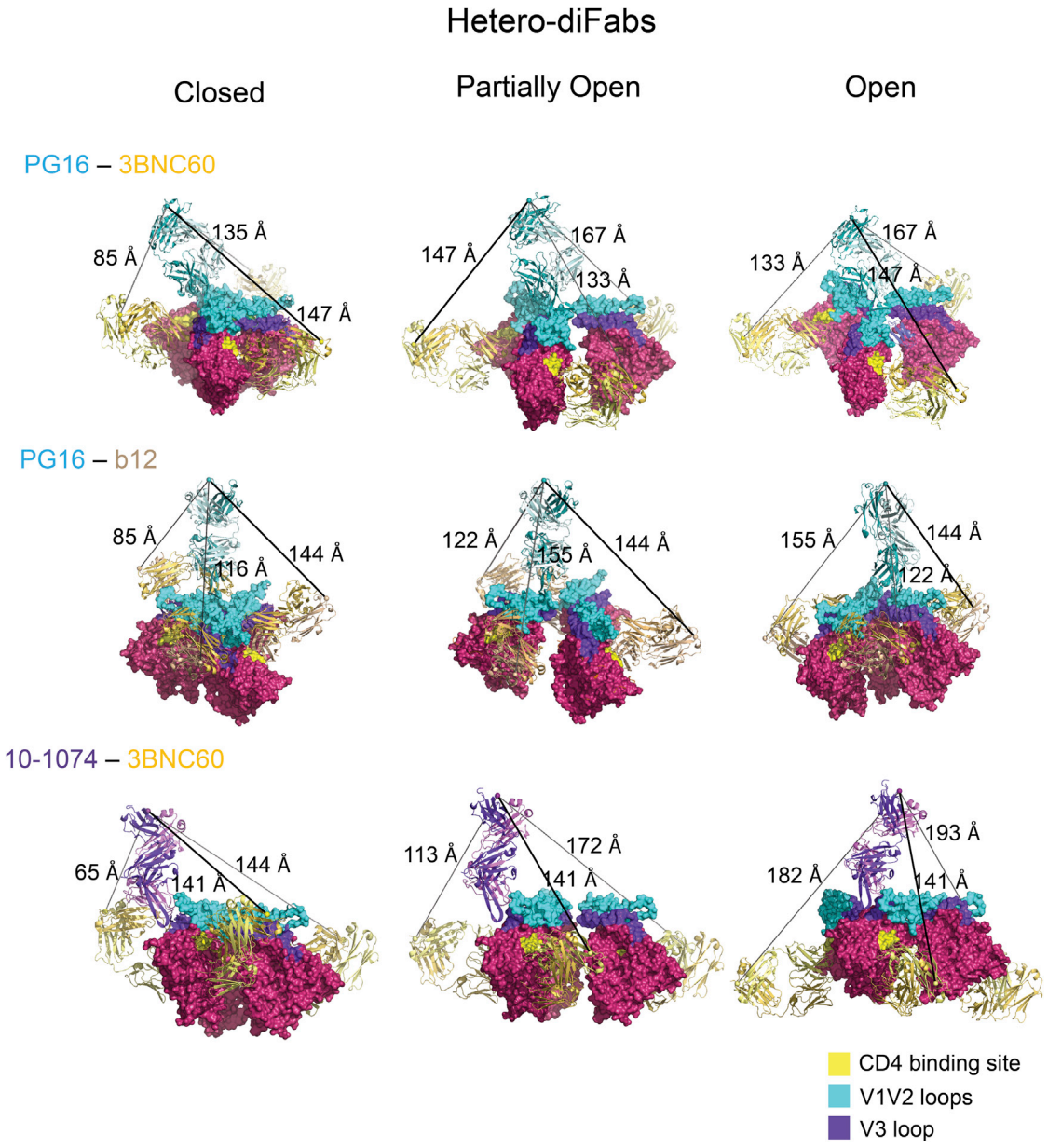

Figure S3. Measured Distances between Hetero-diFabs Bound to HIV-1 Trimer Structures, Related to Figure 3

Fabs from the indicated bNAbs shown bound to the gp120 portions of Env in three conformations: closed, partially open, and open (Liu et al., 2008; Lyumkis et al., 2013; Merk and Subramaniam, 2013; Tran et al., 2012) (see Experimental Procedures and Figure 3). Fabs are shown as ribbons; gp120 subunits are shown as surface representations with V1V2 loops in cyan, V3 in purple, the CD4 binding site in yellow, and the remainder of gp120 in maroon. The distance between the $Cys233_{heavy\ chain}$ carbon-α atoms of adjacent bound Fabs is indicated by a gray line as an approximation of an optimal length for a dsDNA bridge attached to $Cys233_{heavy\ chain}$. Three distances are possible for hetero-diFabs binding to Env trimer. The distance between Fabs bound to the same gp120 subunit (thick line) remains the same in the three trimer conformations.

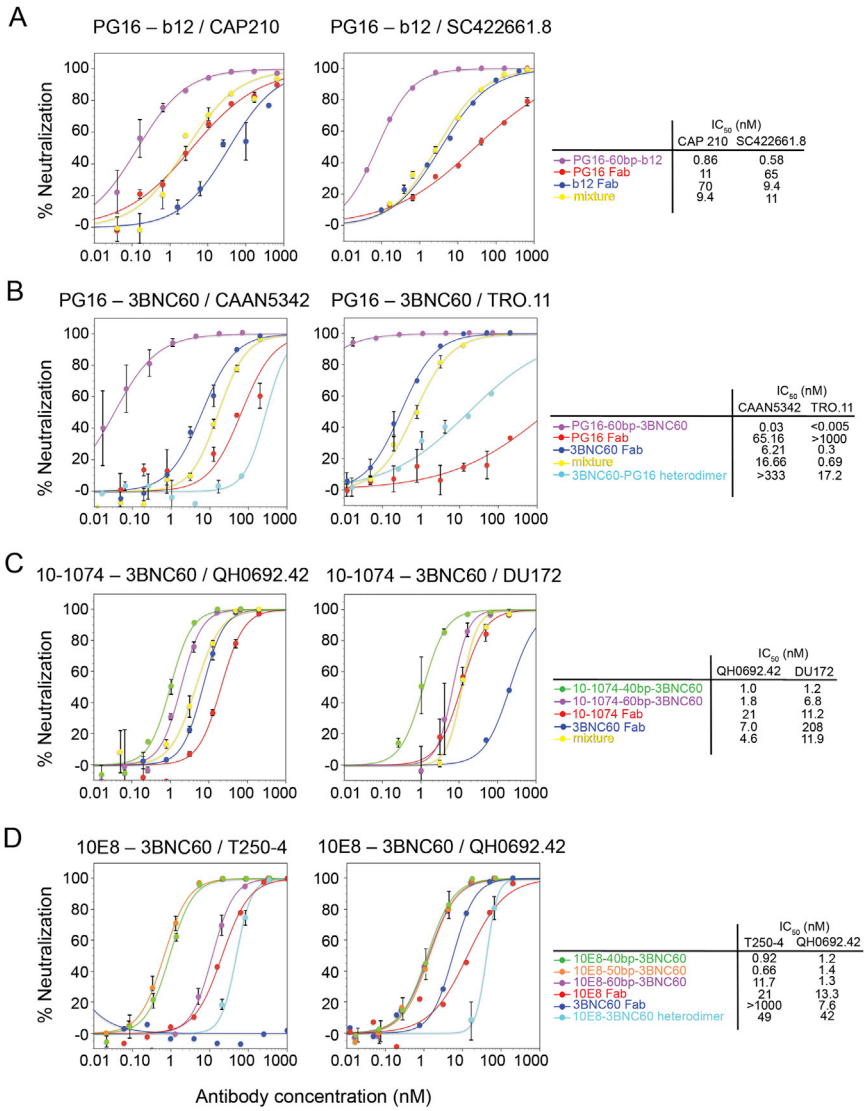

Figure S4. Examples of Neutralization Data for Hetero-diFabs, Related to Figure 4

Neutralization of the indicated viral strains was compared for hetero-diFabs (separated by different dsDNA bridge lengths), each of the parent Fabs alone, a non-covalent mixture of the parent Fabs plus dsDNA, and (when available) the analogous heterodimeric IgG. IC_{50} values are shown on the right. Error bars represent standard deviations of measurements at each concentration.

(A) PG16-60bp-b12 hetero-diFab and controls.

(B) PG16 – 3BNC60 hetero-diFabs and controls.

(C) 10-1074 – 3BNC60 hetero-diFabs and controls.

(D) 10E8 – 3BNC60 hetero-diFabs and controls.

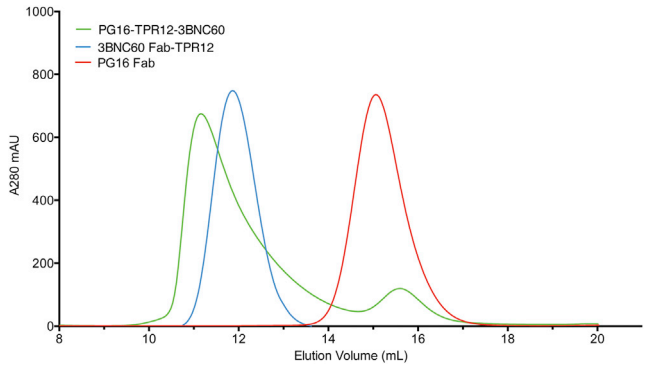

Figure S5. Size Exclusion Chromatography Profiles for PG16-TPR12-3BNC60, Related to Figure 5

SEC runs from which PG16-TPR12-3BNC60 was isolated from fractions 10.3 ml – 11.8 ml. SEC profiles are shown for 3BNC60 Fab-TPR12 and PG16 Fab for comparison. Fractions were assayed by 10% SDS-PAGE (data not shown).